Schistosoma japonicum egg antigens stimulate CD4$^+$ CD25$^+$ T cells and modulate airway inflammation in a murine model of asthma

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Summary

A number of epidemiological and clinical studies have suggested an inverse association between allergy and helminth infection, such as Schistosomiasis. Therefore, we hypothesize that *Schistosoma japonicum* egg antigens, a type of native antigen, can induce production of CD4$^+$ CD25$^+$ T cells with regulatory activity, modulating airway inflammation and inhibiting asthma development. The frequency of CD4$^+$ CD25$^+$ T cells was determined by flow cytometry for mice treated with ovalbumin (OVA), CD25$^+$ depletion/OVA, schistosome egg antigens, schistosome egg antigens/OVA and for control mice. The ability of CD25$^+$ T cells from these mice to suppress T-cell proliferation and cytokine production was investigated both *in vivo* and *in vitro*. Results showed that the CD4$^+$ CD25$^+$ T cells of OVA-treated mice exhibited impaired control of dysregulated mucosal T helper 2 responses compared to the controls ($P < 0{\cdot}05$). Depletion of CD25$^+$ cells accelerated OVA-induced airway inflammation and increased the expression of interleukin (IL)-5 and IL-4. Treatment with schistosome egg antigens increased the number and suppressive activity of CD4$^+$ CD25$^+$ T cells, which made IL-10, but little IL-4. In a murine model of asthma, *S. japonicum* egg antigens decreased the expression of Th2 cytokines, relieved antigen-induced airway inflammation, and inhibited asthma development. Thus, we provided evidence that *S. japonicum* egg antigens induced the production of CD4$^+$ CD25$^+$ T cells, resulting in constitutive immunosuppressive activity and inhibition of asthma development. These results reveal a novel form of protection against asthma and suggest a mechanistic explanation for the protective effect of helminth infection on the development of allergy.

Keywords: asthma; CD4$^+$ CD25$^+$ T cells; IL-10; egg antigen; *Schistosoma japonicum*

Introduction

Strong evidence exists to support the contention that the development of allergies and auto-immunity are influenced by both genetic background and environment/lifestyle.[1,2] The prevalence of allergic diseases such as asthma, the best-documented of the allergic diseases, has increased markedly over the past few decades.[3,4] Hypotheses to explain the increasing prevalence of the dysregulated mucosal T helper 2 (Th2) responses that characterize allergic hyperreactivity have typically been framed in terms of reduced or absent stimulation by Th1-polarizing stimuli. Often called the 'hygiene' hypothesis, the predominant model has suggested that this dramatic increase in allergic disease parallels the greatly reduced exposure to childhood bacterial and viral infections brought about

Abbreviations: BALF, bronchoalveolar lavage fluid; Foxp3, forkhead box P3; LNFP, lacto-*N*-fucopentose; LTNT, lacto-N-tetrose; OVA, ovalbumin; PBMC, peripheral blood monocyte; PBS, phosphate-buffered saline; SEA, soluble egg antigen; *S. japonicum*, *Schistosoma japonicum*; *S. mansoni*, *Schistosoma mansoni*; Treg cell, regulatory T cell.

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by improvements in vaccination and sanitation.[5–7] Yet, this hypothesis virtually ignores the effect of the elimination of chronic Th2-polarizing stimuli during this same time period. Interestingly, many studies have indicated that little allergic disease is found in developing countries where Th2-mediated helminth infection is still endemic. Some epidemiological and clinical studies have suggested an inverse association between helminth infection and allergy.[8–10] However, there is very little data available to explore the mechanism by which helminth infection might protect against allergy. It is clear that the protective effect of helminth infection on allergy development cannot be explained simply in terms of the absence of Th1-inducing stimuli. This has led to a reworking of the hygiene hypothesis in which the counter-regulatory mechanisms induced by all types of inflammatory responses are emphasized.[9,11,12] Prominent among these mechanisms is the regulatory T-cell secretion of immunosuppressive cytokines such as interleukin (IL-)10 and transforming growth factor-β (TGF-β), which regulate Th2 cell-mediated allergic inflammation in the airways.

$CD4^+$ $CD25^+$ regulatory T cells (Treg cells) exhibit a constitutive immunosuppressive activity that was first described in the context of autoimmune disorders.[13,14] They are one of several subsets of regulatory T cells involved in controlling inappropriate or excessive immune activation.[15,16] $CD4^+$ $CD25^+$ T cells have been demonstrated to suppress antigen-specific $CD4^+$ or $CD8^+$ T cell responses directed against allografts[17] and tumours,[18] as well as against bacterial,[19] parasitic[20] and viral[21] antigens or infections. Murine $CD4^+$ $CD25^+$ T cells mediate the suppression of effector T-cell function both *in vitro* and *in vivo* via several mechanisms, requiring either cell–cell contact[22] or the production of immunosuppressive cytokines, such as IL-10[23] and TGF-β.[24] Recent work has shown that the forkhead family transcription factor Foxp3 is critically important for the development and function of regulatory T cells. Lack of Foxp3 leads to development of fatal autoimmune lymphoproliferative disease.[25]

Evidence indicates that chronic *Schistosoma mansoni* infection or exposure to eggs from this helminth down regulates the type 1 immune response and prevents the onset of Th1-mediated diseases such as multiple sclerosis, diabetes mellitus and Graves' hyperthyroidism.[26–28] Other studies have revealed that chronic exposure to *S. mansoni* also down-regulates Th2-mediated atopic diseases.[12,29,30] Moreover, recent reports show *S. mansoni* infection leads to the development of a population of $CD4^+$ $CD25^+$ T cells that make IL-10, but little of the signature Th2 cytokines, and inhibit T cell proliferation and cytokine production.[31,32]

This study used a murine model of asthma to determine if *S. japonicum* egg antigens can induce production of $CD4^+$ $CD25^+$ T cells with immunosuppressive activity and the ability to modulate airway inflammation and inhibit asthma development. We postulate that $CD4^+$ $CD25^+$ T cells generally inhibit potentially pathogenic Th2 responses to allergens, and that this process is deficient in individuals or mice with asthmatic disease.

Materials and methods

Mice and antigen preparations

Six–8-week-old female BALB/c mice were purchased from SLAC Laboratory (Shanghai, China) and bred in our own facilities. All mice were kept in well-controlled animal housing facilities and had free access to tap water and pellet food throughout the experimental period. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals. *S. japonicum* eggs were extracted from the livers of infected rabbit and enriched for mature eggs. Soluble schistosome egg antigen (SEA) was obtained from homogenized *S. japonicum* eggs as described.[33]

Immunizations

S. japonicum egg antigens were used to immunize 10 mice in each of four experimental groups. Each mouse was either intravenously (i.v.) administered 200 μg of SEA, fed with 10 000 dead *S. japonicum* eggs, fed 200 μg of SEA, or injected with SEA and fed SEA or eggs. Depending on the experiment, the immunizations were given once a week for a period of 4 weeks. Control mice were injected with phosphate-buffered saline (PBS).

For allergen sensitization, pulmonary sensitization to ovalbumin (OVA) was performed as previously described.[34] Briefly, in each of the four experimental groups, 10 mice were injected intraperitoneally (i.p.) with 10 μg OVA (Sigma, St. Louis, MO), absorbed to 3 mg of aluminium hydroxide in 0·3 ml of sterile PBS on days 0, 7, and 14. On days 26, 27, and 28 after the initial sensitization, mice were anaesthetized with ketamine anaesthesia (Sigma, 35 mg/kg) and airway challenged intranasally (i.n.) with 100 μg of OVA in 50 μl of PBS. In all experiments, control mice were handled identically and administered saline and aluminium hydroxide i.p and i.n. with PBS while following the same schedule that was used for the experimental animals.

In some of the experiments evaluating *S. japonicum* egg antigen immunotherapy, the murine model of asthma received four injections of SEA (200 μg/mouse) plus oral administration of dead *S. japonicum* eggs (10 000/mouse) for a period of 4 weeks.

Cell depletion

To examine whether Treg cells contribute to the suppression of the T-cell response, mice were depleted of CD25 cells using 500 μg of 7D4, a CD25-specific monoclonal

antibody (mAb; American Type Culture Collection, Manassas, VA) 2 days before immunizing them with OVA. Control mice were injected with an isotype of anti-CD25 mAb.

Cell isolation and flow cytometry

Flow cytometric quantification of cellular subsets was performed using fresh peripheral blood mononuclear cells (PBMC) surface stained with mAbs recognizing anti-CD4–phycoerythrin (PE)-Cy5 and anti-CD25–fluoroscein isothiocyanate (FITC; BD PharMingen, San Diego, CA).

Splenocytes were depleted of CD8 cells using anti-CD8–coupled immunomagnetic beads (Dynal, Brown Deer, CA). CD4 cells were isolated from CD8-depleted splenocytes with a negative $CD4^+$ T cell isolation kit (Miltenyi Biotech, Auburn, CA). $CD4^+$ $CD25^+$ T cells were isolated from the pure, untouched $CD4^+$ T cells using CD25 microbeads (Miltenyi Biotech). The purity of the $CD4^+$ $CD25^+$ population was >85% and the purity of the $CD4^+$ $CD25^-$ population was >90%. Antigen-presenting cells (APC), obtained by isolating the positive fraction of the $CD4^+$ no-touch magnetic sort after depleting $CD8^+$ T cells, were irradiated with 30 Gy.

In vitro *suppression assay*

$CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ T cells were isolated (10 mice/group) and proliferation assays were performed in triplicate by culturing $CD4^+$ $CD25^-$ cells (5×10^4), $CD4^+$ $CD25^+$ T cells (5×10^4) or both populations in 96-well U-bottom plates (0·2 ml/well) with APCs (5×10^4) for 72 hr at 37° in complete medium. Cultures were polyclonally stimulated by soluble anti-CD3 (10 μg/ml) (BD PharMingen) or SEA (50 μg/ml). Supernatants were collected at 72 hr and the production of cytokines was measured, as described below, using Luminex technology. After 3 days, 0·5 μCi/well [^{3}H]thymidine was added. Incorporation of [^{3}H]thymidine was assessed after 16 hr of additional culturing.

Intracellular staining

The cytokine profile of $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells was analysed and compared using anti-IL-4–PE, anti-IL-10–PE, and anti-interferon-γ (IFN-γ)–PE mAbs. Isolated $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells were activated with SEA (100 μg/ml) for 6 hr. Brefeldin A (0·7 μg/ml; Sigma) was added for the last 5 hr. Cells were then washed, fixed and permeabilized (Cytofix/Cytoperm solution; BD PharMingen) and stained with titrated amounts of cytokine-specific antibodies.

To detection the expression of Foxp3 in $CD4^+$ $CD25^+$ T cells, 1×10^6 splenocytes were surface-stained with FITC anti-mouse CD4 mAbs and APC anti-mouse CD25 mAbs after isolation, and subsequently with 0·5 μg PE anti-mouse Foxp3 or PE rat immunoglobulin G2a (IgG2a) isotype controls using Mouse Regulatory T Cell Staining Kit (eBioscience, San Diego, CA).[35]

Cytokine detection

Cytokines in serum, in bronchoalveolar lavage fluid (BALF) and in culture supernatant were analysed using a mouse cytokine multiplex assay (IL-10, IL-4, IL-5, IL-2 and IFN-γ) from R & D Systems, Inc. (Minneapolis, MN). The assay was performed according to the manufacturer's protocol. Briefly, 25 μl of cell supernatant was incubated with antibody-coated beads mix for 16 hr at 4°. The beads were washed twice and incubated with secondary biotinylated antibody mix for 1 hr at room temperature. Streptavidin–PE was added and the mixture was incubated for an additional 30 min at room temperature. The beads were washed three times and then analysed on a Luminex 100 (Luminex, Austin, TX) platform. Cytokine concentrations were calculated from standard curves. A DuoSet enzyme-linked immunosorbent assay (ELISA) development kit for the detection of murine TGF-β was purchased from R & D Systems. TGF-β was assayed according to the manufacturer's instructions. The minimum significant values of these assays were: 10 pg/ml of IL-4, IL-5 and IL-10 and 30 pg/ml of IFN-γ and TGF-β in serum; 3 pg/ml of IL-4 and IL-5 and 20 pg/ml of IFN-γ in BALF; and 2 pg/ml of IL-2.

BALF and histopathology

At 24 hr after the last OVA challenge, mice were deeply anaesthetized by i.p. injection of urethane (15 mg/10 g body weight) (Sigma-Aldrich), immediately after which blood samples were collected and the resulting sera were stored. Airways were washed twice with 1 ml PBS, and the BALF cell differential counts and percentages were determined by Diff-Quik staining of cytospin slides. Two hundred cells per slide were counted. Aliquots of BALF were stored for cytokine measurements.

Immediately after BALF collection, the lungs were fixed in 10% neutral-buffered formalin. Paraffin-embedded sections (4 μm) were stained with haematoxylin and eosin. Tissue-infiltrating inflammatory cells were quantitated by counting the cells in 10 high-power fields (1000×) from 10 similarly sized small bronchioles or 10 similarly sized blood vessels per mouse. Inflammation was scored by three independent blinded investigators. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0–3, as described elsewhere.[36] A value of 0 was assigned when no inflammation was detectable, a value of 1 was assigned for occasional cuffing with inflammatory cells, a value of 2 was assigned when most bronchi or vessels were surrounded by a thin layer (one to five cells thick) of inflammatory cells, and a value

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of 3 was given when most bronchi or vessels were surrounded by a thick layer (more than five cells thick) of inflammatory cells. Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores.

Statistical analysis

Results are expressed as means ± SEM. Comparisons between the two groups of mice were analysed using Mann–Whitney *U*-tests. The statistical analysis was performed by using the SPSS 10.1 program. A value of $P < 0{\cdot}05$ was considered statistically significant.

Results

SEA injection plus oral administration of *S. japonicum* eggs increased the number of $CD4^+$ $CD25^+$ T cells and IL-10 *in vivo*

Thymus-derived endogenous population of $CD4^+$ $CD25^+$ regulatory T cells constitute 5–7% of peripheral $CD4^+$ T cells in naive mice and humans. $CD4^+$ $CD25^+$ regulatory T cells could also be induced at peripheral by infection or administration of antigens. To determine which route of administration could induce an increased number of $CD4^+$ $CD25^+$ T cells, cohorts of female mice were either injected with SEA, orally administered dead *S. japonicum* eggs, orally administered SEA, or treated with SEA plus orally administered eggs once a week for a period of 4 weeks. Control mice were injected with PBS. Only injections of SEA plus oral administration of dead eggs induced a significant increase in the number of $CD4^+$ $CD25^+$ T cells when compared with other methods of administration and with the controls ($P < 0{\cdot}05$) (Fig. 1a). Thus, in the following experiments evaluating *S. japonicum* egg antigen immunotherapy, the murine model of asthma received four injections of SEA (200 μg/mouse) plus oral administration of dead *S. japonicum* eggs (10 000/mouse) for a period of 4 weeks.

The levels of IL-10 and TGF-β, two immunosuppressive cytokines produced by $CD4^+$ $CD25^+$ T cells, were measured in SEA plus oral administration of dead *S. japonicum* eggs mice and controls. In serum, IL-10 was significantly increased in the egg antigen treatment group compared with the controls ($P < 0{\cdot}05$). However, there was no significant difference in the level of TGF-β between the two groups (Fig. 1b).

In vitro $CD4^+$ $CD25^+$ T cells made IL-10 and suppressed proliferation and IL-2 production of $CD4^+$ $CD25^-$ T cells in response to SEA

To study the immunoregulatory capacity of $CD4^+$ $CD25^+$ T cells from *S. japonicum* egg antigen mice, coculture

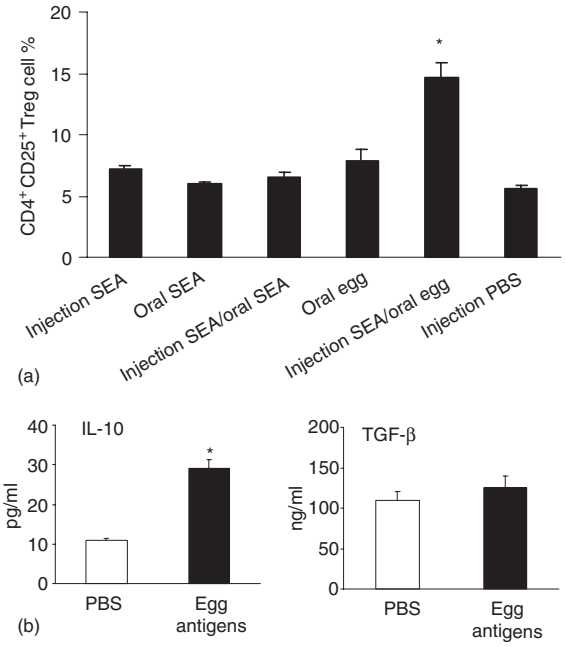

Figure 1. *S. japonicum* egg antigen treatment induces an increase in $CD4^+$ $CD25^+$ T-cell numbers and serum IL-10 *in vivo*. (a) Six groups of BALB/c mice (10/group) were immunized. Each mouse was either i.v. injected with 200 μg of SEA, fed 10 000 dead *S. japonicum* eggs, fed 200 μg of SEA, or injected with SEA and fed SEA or eggs. Control mice were injected with PBS. The immunizations were given once a week for a period of 4 weeks. Twenty-four hr after the last immunization, the mice were killed and PBMCs were surface stained with anti-CD4–PE-Cy5 and anti-CD25–FITC mAbs. The percentage of $CD4^+$ $CD25^+$ T cells among $CD4^+$ T cells was determined by flow cytometry, $^*P < 0{\cdot}05$ compared to all other groups. (b) The levels of IL-10 and TGF-β were measured in SEA plus oral administration of dead *S. japonicum* eggs mice and controls. $^*P < 0{\cdot}05$ compared to PBS mice, the difference of TGF-β was not statistically significant.

experiments were performed using equal numbers of $CD4^+$ $CD25^+$ and autologous $CD4^+$ $CD25^-$ T cells that were stimulated by SEA. The isolated $CD4^+$ $CD25^+$ T cells dramatically decreased the proliferation of $CD4^+$ $CD25^-$ T cells (Fig. 2a).

The ability to inhibit IL-2 production from $CD4^+$ $CD25^-$ T cells was one of the first activities ascribed to $CD4^+$ $CD25^+$ T cells.[37] As expected, inhibition of SEA stimulated $CD4^+$ $CD25^-$ T-cell proliferation by $CD4^+$ $CD25^+$ T cells from *S. japonicum* egg immunized mice was associated with a reduction in antigen-induced IL-2 secretion; the $CD4^+$ $CD25^+$ T cells themselves did not produce IL-2 in response to SEA (Fig. 2b).

In addition, the cytokine profile of $CD4^+$ $CD25^+$ T cells was determined. After 4 hr of SEA stimulation, $CD4^+$ $CD25^+$ T cells showed enhanced IL-10 production compared with the $CD4^+$ $CD25^-$ population. In contrast, $CD4^+$ $CD25^+$ T cells displayed a marked decrease of IFN-γ

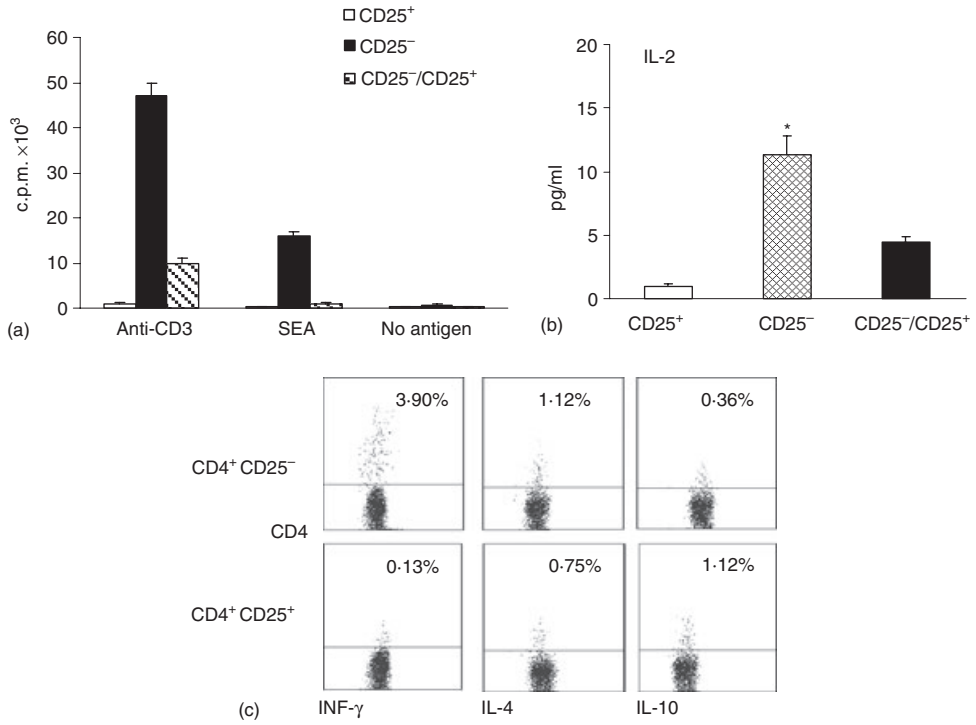

Figure 2. Functional analysis of $CD4^+$ $CD25^+$ T cells from *S. japonicum* egg antigen mice. (a) $CD4^+$ $CD25^+$ T cells decreased the proliferation of $CD4^+$ $CD25^-$ T cells. Twenty-four hr after the last antigen administration, mice were killed and proliferation assays were performed in triplicate by culturing freshly isolated $CD4^+$ $CD25^-$ cells (5×10^4), $CD4^+$ $CD25^+$ T cells (5×10^4) or both populations in 96-well U-bottom plates (0·2 ml/well) with APCs (5×10^4) for 72 hr at 37°. Cultures were polyclonally stimulated by soluble anti-CD3 (10 μg/ml) or SEA (50 μg/ml). After 3 days, 0·5 μCi/well [^{3}H] thymidine was added. Incorporation of [^{3}H]thymidine was assessed after 16 hr of additional culturing. The data represent the mean c.p.m. determined for each of the 10 individual animals per experimental group. (b) $CD4^+$ $CD25^+$ T cells suppress the IL-2 production of $CD4^+$ $CD25^-$ T cells. $CD4^+$ $CD25^-$ T cells (5×10^4) alone or cocultured with $CD4^+$ $CD25^+$ T cells (5×10^4) were stimulated with 100 μg/ml SEA for 48 hr and supernatants were collected for mouse cytokine multiplex assay. $^*P < 0{\cdot}05$ compared to all other mice. (c) Cytokine profiles of $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells. Intracellular cytokine production of $CD4^+$ $CD25^+$and $CD4^+$ $CD25^-$ T cells was determined by PE conjugated anti-IL-4, anti-IL-10, and anti-IFN-γ mAbs after 6 hr of SEA stimulation.

production when compared with $CD4^+$ $CD25^-$ T cells. Both populations synthesized a certain amount of IL-4 (Fig. 2c).

In the murine model of asthma, treatment of mice with *S. japonicum* egg antigens induced $CD4^+$ $CD25^+$ cells with decreased suppressive activity

The percentage of $CD25^+$ T cells in the total $CD4^+$ T cell population was measured in OVA mice, egg antigens mice, egg antigens/OVA mice and control mice. In control mice, the mean percentage of $CD4^+$ $CD25^+$ T cells was 7·43 ± 0·95% of all $CD4^+$ T cells. The percentage in OVA mice was 7·03 ± 1·07% (Fig. 3a). This result showed that the mean percentage of $CD4^+$ $CD25^+$ T cells from all $CD4^+$ T cells was not significantly decreased in OVA mice ($P = 0{\cdot}085$). However, $CD4^+$ $CD25^-$ T-cell proliferation mixed with autologous $CD4^+$ $CD25^+$ T cells was higher in OVA mice (suppression <48%) as compared with controls (suppression >63%) (Fig. 3b). Meanwhile, the mean percentage of $CD4^+$ $CD25^+$ T cells were significantly increased in egg antigens mice and egg antigens/OVA mice (Fig. 3a). In addition, $CD4^+$ $CD25^-$ T-cell proliferation when mixed with autologous $CD4^+$ $CD25^+$ T cells was lower in *S. japonicum* egg mice (suppression <81%) than in the controls (suppression >63%) (Fig. 3b).

It was important to examine whether the loss of regulatory function in OVA mice was caused by a decrease in $CD4^+$ $CD25^+$ T-cell suppressive function or to an increase in the resistance of activated $CD4^+CD25^-$ responder T cells to inhibition. Thus, the suppressive ability of $CD4^+$ $CD25^+$ T cells from OVA mice, *S. japonicum* egg mice and the controls was compared. Co-culture experiments were performed using equal numbers of $CD4^+$ $CD25^+$ T cells from OVA mice, *S. japonicum* egg mice or the controls and $CD4^+$ $CD25^-$ T cells from control mice, which were stimulated by anti-CD3. Strikingly, OVA mice showed decreased suppressive activity in regulatory $CD4^+$ $CD25^+$ T cells when compared with controls

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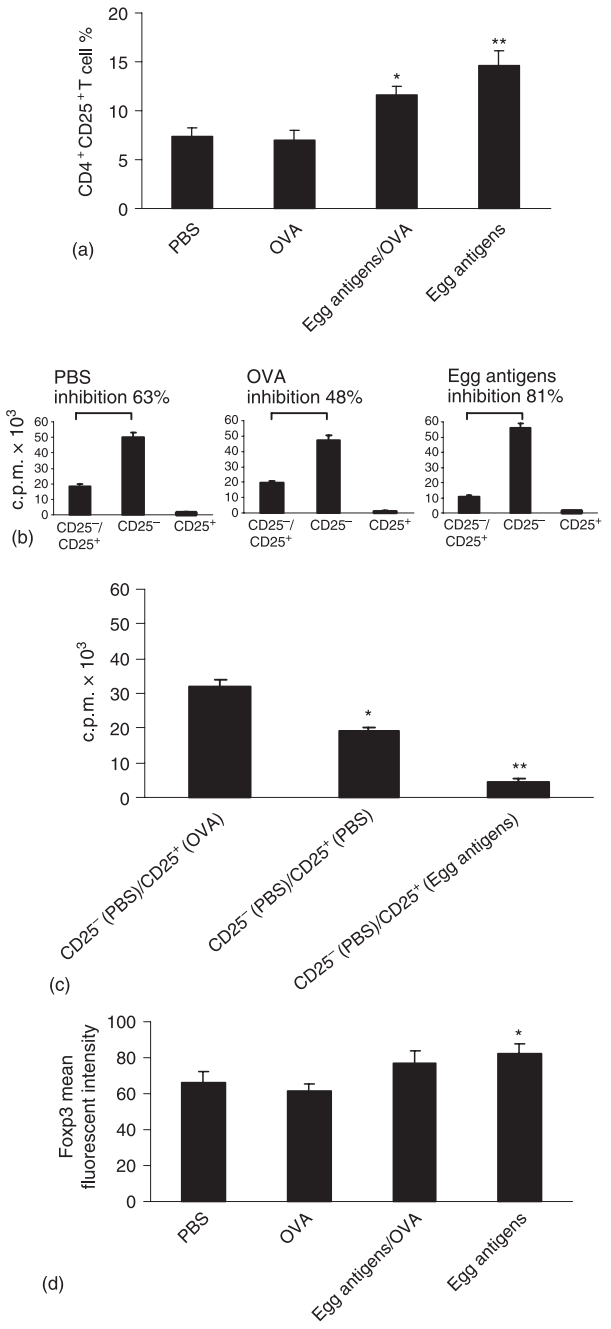

Figure 3. Characterization of $CD4^+$ $CD25^+$ T cells in mice treated with PBS, OVA and *S. japonicum* egg antigen. (a) The percentage of $CD4^+$ $CD25^+$ T cells among $CD4^+$ T cells was determined by flow cytometry and compared with OVA mice and PBS mice. Twenty-four hr after final treatment with antigens, mice were killed and splenocytes were stained with anti-CD4 FITC and anti-CD25 PE-Cy5 and FACS analysed. $^*P < 0{\cdot}05$ compared to OVA mice; $^{**}P < 0{\cdot}001$ compared to PBS mice. The data represent the mean number of $CD4^+$ $CD25^+$ T cells in the 10 individual animals per experimental group. (b) The suppressive efficacy of $CD4^+$ $CD25^+$ T cells mediating suppression of $CD4^+$ $CD25^-$ responder cell proliferation is altered in different antigen-treated mice. $CD4^+$ $CD25^+$ T cells were activated with soluble anti-CD3 mAb and tested for their ability to proliferate in 1 : 1 cocultures of $CD4^+$ $CD25^+$ and autologous $CD4^+$ $CD25^-$ T cells. (c) The suppressive ability of $CD4^+$ $CD25^+$ T cells was compared between mice treated with different antigens. $CD4^+$ $CD25^+$ T cells from OVA mice, PBS mice or egg antigens mice were cocultured with $CD4^+$ $CD25^-$ T cells and APCs from PBS mice (ratio of 1 : 1 : 1) activated with soluble anti-CD3 mAb. $^*P < 0{\cdot}05$ compared to $CD4^+CD25^+$ T cells from OVA mice, $^{**}P < 0{\cdot}05$ compared to other groups. (d) FACS analysis of Foxp3 mean fluorescence intensity in $CD4^+$ $CD25^+$ T cells from PBS-treated mice, OVA-treated mice and *S. japonicum* egg antigen-treated mice. Splenocytes were stained at the cell surface with mAbs against CD4 and CD25, followed by intracellular staining for FoxP3. Representative expression of CD25 and FoxP3 on gated $CD4^+$ cells. $^*P < 0{\cdot}05$ compared to OVA mice.

($P < 0{\cdot}05$), but *S. japonicum* egg antigens treatment significantly increased suppressive activity in regulatory $CD4^+$ $CD25^+$ T cells when compared with controls ($P < 0{\cdot}01$) (Fig. 3c). These data indicated that the primary regulatory defect was in the function of $CD4^+$ $CD25^+$ T cells isolated from the OVA mice.

Until very recently[38] there were no reports of surface markers that are uniquely expressed on suppressive $CD4^+$ $CD25^+$ T cells, making identification and quantification of this population very difficult. However, the intracellular transcription factor Foxp3 is now known to be functionally relevant to the immunosuppressive activity of $CD4^+$ $CD25^+$ T cells in both mice and humans.[25,36,39] In this regard, the mean fluorescent intensity of the $Foxp3^+$ population was measured in $CD4^+$ $CD25^+$ T cells. Our results showed that Foxp3 was highly expressed in fresh $CD4^+$ $CD25^+$ T cells from *S. japonicum* egg antigen-treated mice with lower expression observed in OVA-treated mice and controls (Fig. 3d).

Antigen-induced inflammatory cell recruitment into the airways is decreased with *S. japonicum* egg antigen treatment, while depletion of $CD25^+$ cells in naïve mice increased the inflammatory response

To examine whether $CD4^+$ $CD25^+$ T cells contribute to the regulation of OVA-induced inflammation, $CD25^+$ cells were depleted 2 days before first antigen immunization(Fig. 4a). These depleted $CD25^+$ cells in naïve mice were mainly endogenous Treg cells. The effect of $CD4^+$ $CD25^+$ T cells on OVA-induced airway inflammation was ascertained by determining the inflammatory score and the cellular composition in BALF fluid in four groups of mice. As shown in Table 1, the total number of cells and the number of macrophages, neutrophils and eosinophils in BALF were significantly greater in the OVA/$CD25^+$ cell depletion group than in the OVA group ($P < 0{\cdot}05$ for total number of cells, macrophages and eosinophils, $P < 0{\cdot}01$ for neutrophils). Lung lymphocytes and eosinophils were significantly decreased in mice exposed to *S. japonicum* egg antigen/OVA. No neutrophils

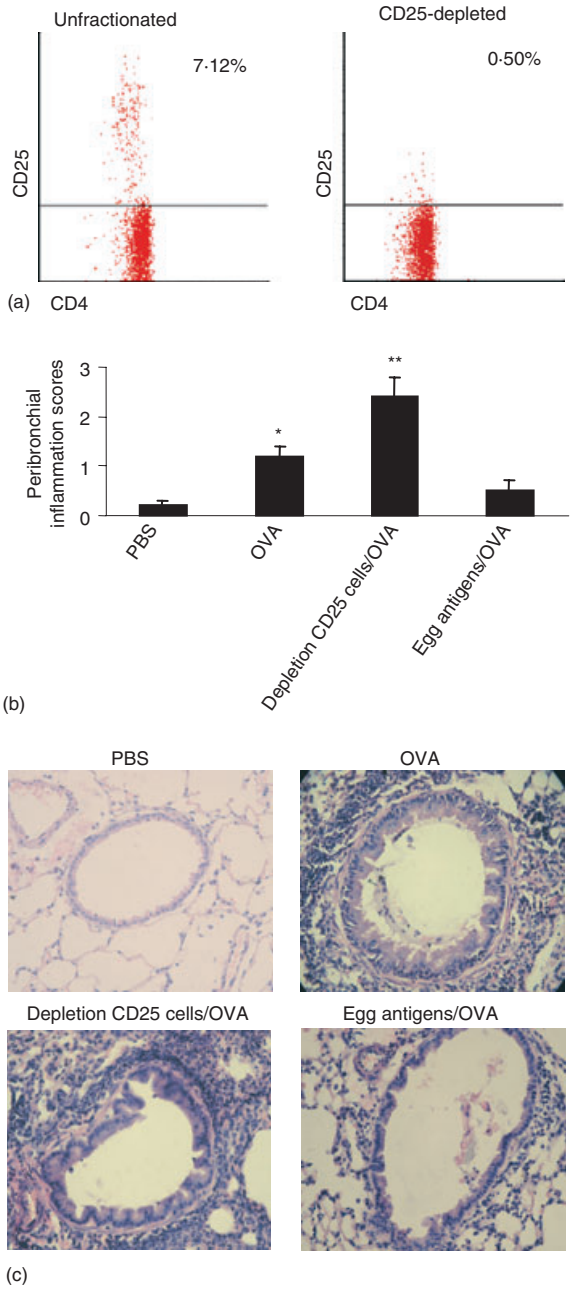

Figure 4. Quantification of histological inflammation in mice treated with PBS, OVA or *S. japonicum* egg antigen. (a) Depletion of $CD4^+$ $CD25^+$ T cells in a murine model of asthma. $CD4^+$ $CD25^+$ T cells were depleted from BALB/c mice using anti-CD25 mAb before OVA sensitization as described in Methods. The efficiency of depletion was evaluated by FACS with anti-CD4-PE-Cy5 and anti-CD25-FITC. (b) Mean peribronchial inflammation scores, $^*P < 0{\cdot}01$ compared to *S. japonicum* egg antigen/OVA mice; $^{**}P < 0{\cdot}05$ compared to OVA mice. (c) Allergen-induced pulmonary inflammation. PBS did not induce pulmonary inflammation. OVA induced a predominantly lymphocytic infiltrate with some eosinophilia. Before depletion of CD25 cells, OVA induced a significant lymphocytic, eosinophilic and neutrophilic infiltration with some necrosis. Pulmonary inflammation induced by OVA is markedly diminished by coadministration of SEA and dead *S. japonicum* eggs.

or eosinophils were observed in the BALF of the control group.

The number of inflammatory cells per length of basement membrane of the airways was used as a measure of the amount of infiltration around the airways. The OVA/$CD25^+$ cell depletion group produced a significant increase in the number of infiltrated inflammatory cells compared with the OVA group ($P < 0{\cdot}05$) (Fig. 4b) along with increased allergen-induced pulmonary inflammation (Fig. 4c). Mice that received *S. japonicum* egg antigens had markedly fewer tissue-infiltrating inflammatory cells and diminished peribronchial inflammatory response in the lungs (Fig. 4b, c). Therefore, those mice that received *S. japonicum* egg antigens were protected from inflammation and asthma development.

In a murine model of asthma, the expression of Th2 cytokines decreased in mice treated with *S. japonicum* egg antigens, while depletion of $CD25^+$ cells in naïve mice increased Th2 cytokine expression

To assess the potential role of $CD4^+$ $CD25^+$ T cells in antigen induced Th1 or Th2 response, the protein levels of IFN-γ, IL-5 and IL-4 in BALF and in serum were quantitated in $CD25^+$ cell-depleted OVA mice. In serum, IL-5 was significantly increased in the OVA/$CD25^+$ cell depletion group compared with the OVA group ($P < 0{\cdot}05$). Instillation of OVA alone resulted in significant elevation of IL-4 ($P < 0{\cdot}001$) and IL-5 ($P < 0{\cdot}05$) concentrations in serum compared with controls. No significant difference in the level of IL-4 in serum was found between the OVA and OVA/$CD25^+$ cell depletion groups (Fig. 5a).

In BALF, IL-5 was significantly increased in the OVA/$CD25^+$ cell depletion group compared with the control ($P < 0{\cdot}001$) and OVA ($P < 0{\cdot}05$) groups. The OVA/$CD25^+$ cell depletion group also showed an increase in IL-4 in BALF compared with other groups, although these differences were not statistically significant (Fig. 5b). Interestingly, there was no difference in the level of IFN-γ in either BALF or serum samples from any of the experimental groups shown in Fig. 5(a and b), which indicates that $CD4^+$ $CD25^+$ T cells play an important role in modulating excessive activation of effector Th2 cells.

The potential role of *S. japonicum* egg antigen treatment in OVA-induced Th1 or Th2 response in a murine model of asthma was assessed by quantitating protein levels of IFN-γ, IL-5 and IL-4 in BALF and in serum. In serum, IL-5 was significantly decreased in the OVA/*S. japonicum* egg antigen group compared with the OVA ($P < 0{\cdot}05$) group. A greater decrease in IL-4 was also observed in the OVA/*S. japonicum* egg antigen group than in the OVA group ($P < 0{\cdot}001$) (Fig. 5a). In BALF, IL-4 was significantly decreased in the OVA/*S. japonicum* egg antigen treatment group compared with the OVA group

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Table 1. Cellular profile in bronchoalveolar lavage fluid

	PBS	OVA	OVA/ depletion CD25 cells	OVA/ egg antigens
Total cells ($\times 10^4$/ml)	11·5 ± 1·3	24·2 ± 12·5	35·6 ± 3·2*	17·2 ± 4·3
Macrophage ($\times 10^4$/ml)	11·3 ± 1·6	12·8 ± 8·3	19·2 ± 3·2*	12·6 ± 3·2
Lymphocyte ($\times 10^4$/ml)	0·0 ± 0·0	6·9 ± 3·1	7·7 ± 2·1	3·6 ± 0·2*
Neutrophil ($\times 10^4$/ml)	0·0 ± 0·0	0·8 ± 0·2	2·3 ± 0·1**	0·2 ± 0·1
Eosinophil ($\times 10^4$/ml)	0·0 ± 0·0	3·7 ± 1·8	6·4 ± 1·6*	0·8 ± 0·2**

Four groups (10/group) of mice were intratracheally administered PBS, OVA, OVA after depletion of CD25 cells, or OVA with *S. japonicum* egg antigen treatment for 4 week. BALF was conducted 24 h after the last intratracheal instillation. Total cell counts were determined on fresh BALF fluid and differential cell counts were assessed with Diff-Quik-staining. *$P < 0·05$ compared to OVA mice; **$P < 0·01$ compared to OVA mice.

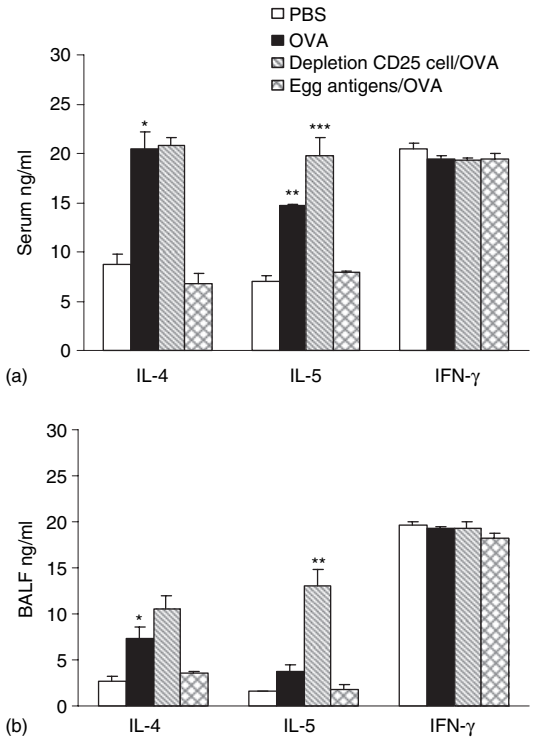

Figure 5. Luminex measurements of IFN-γ, IL-5 and IL-4 protein in BALF and serum of PBS mice, OVA mice, depletion CD25 cells/OVA mice, and *S. japonicum* egg antigen/OVA mice. (a) Measurement of cytokines in serum, *$P < 0·001$ compared to PBS or *S. japonicum* egg antigen/OVA mice; **$P < 0·05$ compared to PBS or *S. japonicum* egg antigen/OVA mice; ***$P < 0·05$ compared to OVA mice. (b) Measurements of cytokine in BALF, *$P < 0·05$ compared to PBS or *S. japonicum* egg antigen/OVA mice; **$P < 0·05$ compared to OVA mice.

($P < 0·05$). The results indicated that IL-5 was also lower in the OVA/*S. japonicum* egg antigen treatment group, although this difference was not statistically significant (Fig. 5b). No differences were found in the level of IFN-γ in BALF or in serum among the experimental groups (Fig. 5a and b).

Discussion

The development of asthma is influenced by both genetic and environmental factors. There is evidence suggesting that infection may be one such environmental modifier in that it plays a role in preventing the onset of this autoimmune condition in rodent models and possibly also in humans.[27,40] The results of this study demonstrated that specific native antigens, *S. japonicum* egg antigens, were able to induce a population of $CD4^+$ $CD25^+$ T cells which elicited immunosuppressive activity in the mice, thereby mediating asthma prevention. Our results showed that $CD4^+$ $CD25^+$ T cells in a murine model of asthma displayed impaired regulation of antigen-specific Th2 cell-mediated allergic inflammation. On the other hand, injection of SEA plus oral administration of *S. japonicum* eggs induced a significant increase of $CD4^+$ $CD25^+$ T cells with immunosuppressive activity. *S. japonicum* egg antigens treatment also increased the production of IL-10 and decreased Th2 cell-mediated allergic inflammation in mice. These findings may provide a novel approach for protection against asthma and suggest a mechanistic explanation for the protective effect of helminth infection on the development of allergy.

Allergic asthma is characterized by airway hyperresponsiveness and mucosal inflammation mediated by $CD4^+$ Th2 cells. That these events arise as a consequence of a defect in immune regulation is implied from the observation that lung mucosal immune responses are normally tightly regulated.[41] Although the difference in $CD4^+$ $CD25^+$ T-cell number between OVA sensitized mice and the controls was not significant, $CD4^+$ $CD25^+$ T cells in OVA mice showed a decrease in suppressive activity when compared to controls. Allergic inflammation is characterized by a prominent eosinophil infiltrate. In OVA mice, Th2 cell-mediated antigen-induced eosinophil recruitment into the airways was significantly increased.[42] In addition, it was found that in OVA mice depleted of $CD25^+$ T cells, Th2 cell-mediated allergic inflammation was up-regulated,

antigen-induced eosinophil, as well as neutrophil recruitment was increased, and OVA induced IL-4 and IL-5 production was also increased. These results indicate that $CD4^+$ $CD25^+$ T cells in the lung suppressed type 2 immune responses, and displayed a defect in down-regulation of Th2 cell-mediated allergic inflammation in the airways. Other reports have shown $CD4^+$ $CD25^+$ T cells can modulate T helper cell differentiation toward the Th2 type[43] but have no effect on the development of bronchial hyperreactivity.[44,45]

Consistent with *S. mansoni* infection leading to the development of $CD4^+$ $CD25^+$ T cells[30,31] *S. japonicum* egg antigens treatment induced a significant increase of $CD4^+$ $CD25^+$ T cells in BALB/c mice after a combination of SEA injection and oral administration of dead *S. japonicum* eggs. Following T-cell receptor engagement, $CD4^+$ $CD25^+$ T cells can suppress the activation and proliferation of other $CD4^+$ and $CD8^+$ T cells in an Ag-non-specific manner[22,46,47] partly by inhibiting IL-2 production.[36] IL-2 is essential for $CD4^+$ $CD25^+$ T-cell function and plays a crucial role in the maintenance of natural immunological self-tolerance.[48,49] Neutralization of circulating IL-2 by anti-IL-2 mAb has been shown to elicit autoimmunity in BALB/c mice.[50] Our results revealed that, *in vitro*, $CD4^+$ $CD25^+$ T cells from *S. japonicum* egg-immunized mice significantly decreased the proliferation of $CD4^+$ $CD25^-$ T cells from both *S. japonicum* egg-immunized mice and controls. Furthermore, when compared with $CD4^+$ $CD25^+$ T cells from PBS or OVA mice, $CD4^+$ $CD25^+$ T cells from *S. japonicum* egg antigen-treated mice showed increased suppressive activity in response to anti-CD3. $CD4^+$ $CD25^+$ T cells from *S. japonicum* egg antigen-treated mice also significantly decreased the proliferation of autologous $CD4^+$ $CD25^-$ T cells and the production of IL-2, when stimulated with SEA. The regulatory activity of Treg cells appears to stem from their expression of Foxp3, a transcription factor that acts as an important regulator of the Treg phenotype.[51] Indeed, we found that Foxp3 was highly expressed in $CD4^+$ $CD25^+$ T cells from *S. japonicum* egg antigen-treated mice compared to expression in OVA-treated mice and controls. This finding is consistent with the data which showed $CD4^+$ $CD25^+$ T cells from *S. japonicum* egg antigen-treated mice possessed increased suppressive activity.

It has been reported that Th1 responses are more prone to regulation by $CD4^+$ $CD25^+$ T cells than Th2 responses[52]. Nevertheless, $CD4^+$ $CD25^+$ T cells can suppress Th2 maturation,[53] possibly by inhibiting IL-4 production[54] and the development of pulmonary eosinophilic inflammation.[55] In this study, the *S. japonicum* egg antigen-treated murine model of asthma showed a decrease of Th2 cytokine (IL-4 and IL-5) expression, but not of Th1 cytokines (IFN-γ), *in vivo* and in the airways. Other results indicated that *S. japonicum* egg antigen treatment did not impair Th1 cell-mediated immune response *in vivo*. This observation is consistent with a significant increase in $CD4^+$ $CD25^+$ T cells and a relief of antigen-induced airway inflammation.

The requirement for IL-10 is critical in several important human diseases, including schistosomiasis, wherein marked increases in host morbidity and mortality are observed when IL-10 levels are low or absent.[56–58] In murine *S. mansoni* infection, IL-10 reduces hepatocyte damage induced by the eggs of the parasite, is essential for maintenance of a non-lethal chronic infection and inhibits inappropriate immune responses in experimental models.[59,60] $CD4^+$ $CD25^+$ T cells secrete immunosuppressive cytokines like IL-10 and TGF-β.[23,24] We found that in serum IL-10, instead of TGF-β, was significantly increased in the egg antigen-treated group compared with the controls. Thus, in this study and in murine *S. mansoni* infection, $CD4^+$ $CD25^+$ T cells were, at least in part, an important contributor to IL-10 production. Thus, blocking IL-10 or modulating $CD4^+$ $CD25^+$ T cells could control helminth infection.[61,62]

Previous studies have shown that during helminthiasis, Lacto-*N*-tetrose (LTNT), a sugar present in many kinds of helminths, and lacto-*N*-fucopentose (LNFP III), found in *S. mansoni*, act as inducing factors of both the type 2 response and production of IL-10.[63] Therefore, the ability of IL-10 to then inhibit the release of histamine and other mediators by cord blood mast cells could be the mechanism behind the inhibition of the skin prick test reaction in schistosomiasis.[64] A study evaluating children living in a *S. haematobium* endemic area in Africa showed high levels of IL-10 and a decreased skin prick test response, suggesting that parasite-induced IL-10 may be involved in that process.[65] Here, we provided evidence demonstrating that induction of IL-10-producing $CD4^+$ $CD25^+$ T cells by *S. japonicum* egg antigens treatment might down-regulate the immediate immune response in a murine model of asthma by inhibiting IL-4 and IL-5 production.

It appears that *S. japonicum* egg antigens treatment, similar to helminth infections[66] can produce regulatory T cells and mediate protection against allergic and autoimmune disorders. The identification of parasite molecules that induce protection against auto-immune and allergic diseases is and will be a challenge to researchers who work in this field, which could lead to the development of new perspectives for the prevention and therapy of asthma or other excessive-immune disorders.

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